VIROLOGY 225, 82–96 (1996)
ARTICLE NO. 0577

Scaffolding Mutants Identifying Domains Required for P22 Procapsid

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Received December 11, 1995; accepted August 13, 1996

Assembly of the icosahedral shells of the dsDNA bacteriophages, herpesviruses, and adenoviruses requires proteins not found in the mature virion, termed scaffolding proteins. The bacteriophage P22 precursor procapsid contains approximately 300 scaffolding molecules within a shell composed of 420 coat protein subunits. Though nonsense mutants are common, few mutants affecting the functions of the scaffolding protein have been recovered. We report here the isolation and characterization of new missense mutants unable to form infectious virions under restrictive conditions. These mutant scaffolding subunits were competent for protein folding and capsid assembly under restrictive conditions. Two mutants were defective in assembly into the procapsid of the portal complex, which serves as the channel through which DNA is packaged. These mutations may identify a region of the scaffolding protein required for interaction with the portal subunits. Two mutants in a different region of the sequence were impaired in scaffolding release from the procapsid both *in vivo* and *in vitro*. These mutations may identify a new domain required for scaffolding release. Scaffolding release appeared to be required for capsid expansion; in turn, scaffolding release seemed to depend upon the presence of a portal. This may help to order the pathway of events in phage maturation. © 1996 Academic Press, Inc.

INTRODUCTION

The first product of assembly of the double-stranded DNA phages (Casjens and Hendrix, 1988) as well as the herpesviruses (Sherman and Bachenheimer, 1988; Lee *et al.,* 1988) and adenoviruses (D'Halluin *et al.,* 1978; Edvardsson *et al.,* 1976) is a precursor capsid that serves as a substrate for DNA packaging. Formation of these precursors requires large numbers of scaffolding molecules, proteins that are not found in the mature virions. Upon the commencement of DNA packaging, all the scaffolding molecules exit the capsid, being either proteolysed into small fragments or released intact. In the absence of scaffolding the coat proteins of dsDNA phages (Hendrix, 1985; Casjens and Hendrix, 1988) and herpesvirus (Desai *et al.,* 1994; Matusick-Kumar *et al.,* 1994; Thomsen *et al.,* 1994; Tatman *et al.,* 1994) form aberrant structures, demonstrating that scaffolding proteins play an essential role in morphogenesis.

Many functions have been envisioned for scaffolding proteins (Kellenberger, 1990; King *et al.,* 1980; Casjens and Hendrix, 1988). They could serve as a core around which the coat proteins can polymerize to form a closed shell of the correct size; they may function to exclude cytoplasmic proteins from within the capsid (Earnshaw and Casjens, 1980); they may function in the recruitment or organization of the portal proteins needed for DNA packaging and injection (Murialdo and Becker, 1978a; Earnshaw and Casjens, 1980; Bazinet and King, 1988); or they may be directly involved in the packaging of the initial coils of condensed DNA within the procapsid (King and Chiu, 1996). If scaffolding molecules serve a number of these functions, the protein may play a complex role in procapsid assembly and DNA packaging.

The assembly pathway of the bacteriophage P22 procapsid which has been studied *in vitro* does not begin with the assembly of a scaffolding core (Prevelige *et al.,* 1988). Instead, assembly appears to proceed by the serial addition of both coat and scaffolding subunits onto the edges of a growing shell (Prevelige *et al.,* 1993). While the scaffolding proteins of T4 (Traub and Maeder, 1984; van Driel and Couture, 1978b) and herpesvirus (Newcomb and Brown, 1991; Preston *et al.,* 1994) are capable of forming naked cores, the *in vivo* pathway of these viruses is believed to also involve copolymerization (Kellenberger, 1990; Thomsen *et al.,* 1995). This implies that the scaffolding proteins have a more active role in the assembly of viral coat subunits into procapsids, one that may involve concerted conformational changes in both coat and scaffolding proteins.

Assembly of a functional procapsid requires more than correct polymerization of the coat protein. P22 procapsids contain minor components in addition to the 420 coat subunits and approximately 300 molecules of scaffolding protein. The portal protein, present in 12 copies per procapsid (Bazinet *et al.,* 1988), comprises the channel through which the DNA is packaged. The portal complex is located at one fivefold vertex of the procapsid

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and serves as the site for tail attachment in the mature phage (Hartweig *et al.,* 1986). The procapsid also includes three pilot proteins required for DNA injection into the host cell (Botstein *et al.,* 1973; Hoffman and Levine, 1975a,b; Bryant and King, 1984). Their location within the capsid is not known, although their low copy number, 10–20 per procapsid (Casjens and King, 1974), implies that they can only be present at a few specialized positions. They might all be located at the portal vertex or in one or two copies at each vertex (Thomas and Prevelige, 1991). Incorporation of these proteins is thought to be an early step in assembly, as neither the portal (Poteete *et al.,* 1979; Fuller and King, 1982) nor the pilot proteins (Jarvik and Botstein, 1973; Thomas and Prevelige, 1991) can be added onto completed procapsids.

Incorporation of these minor proteins may be an additional function of the scaffolding protein. Structures formed by phage coat proteins in the absence of scaffolding lack portals and other minor proteins (Earnshaw and King, 1978; Ray and Murialdo, 1975; van Driel and Couture, 1978a). Genetic evidence from bacteriophage lambda indicates that the presence of the lambda scaffolding protein is required for the formation of the dodecameric portal ring, the first step in procapsid assembly (Murialdo and Becker, 1978a; Murialdo, 1979; Kochan and Murialdo, 1983). A role for scaffoldings in procapsid initiation and portal insertion implies that scaffolding molecules must possess sites for interaction with portal protein in addition to coat and other scaffolding molecules. This idea also suggests that scaffolding molecules may play different roles at different times during the assembly process.

Another distinct process in capsid assembly is capsid maturation, during which the scaffolding is released, the DNA is packaged, and the coat protein undergoes conformational changes resulting in expansion and angularization of the capsid (Prasad *et al.,* 1993). The signaling pathway for initiation of this lattice expansion has not been defined. The conformational changes in the coat protein may be induced through interactions with the portal, with the exiting scaffolding, or by direct interactions with the entering DNA.

In order to exit, the scaffolding must cease its binding to the coat protein, suggesting that the scaffolding molecules or coat subunits must at least undergo some sort of conformational change to be released from the procapsid. While this release can be induced *in vitro* by the denaturant GuHCl (Fuller and King, 1981; Prevelige *et al.,* 1988), this cannot be the mechanism used within the cell. When DNA packaging is blocked by mutations in the DNA packaging proteins, the cell accumulates unexpanded procapsids which still contain scaffolding (King *et al.,* 1973). Perhaps the scaffolding protein can sense the entry of DNA; if so, an additional interaction site with DNA might exist.

It has proven difficult to study these processes *in vivo,* since the steps in capsid assembly are rapid and tightly coupled. The order of events involved in phage maturation is still uncertain. To help elucidate these functions, we set out to isolate mutants in the scaffolding gene and determine their effects on scaffolding function *in vivo.*

MATERIALS AND METHODS

Bacteria

All cell lines used were derivatives of *Salmonella typhymurium* LT2. The suppressor minus host DB7136 (*leu amA414, his amC525*) and the two suppressor plus derivatives DB7155 supE20(Gln) and DB7156 supF20(Tyr) that were used to propagate amber mutants have been described by Winston *et al.* (1979).

Phage

The isolation of mutants in gene *8* (scaffolding) *8tsN102, 8csRN26D,* and gene *5* (coat) *5cs577* and *5cs567* has been described by Jarvik and Botstein (1975). Other mutants used were gene *8amN123* and *8amN26* (Botstein *et al.,* 1972). The U100 mutant series, including *cs* and *ts* mutants, and the U200 series amber mutants were isolated in the laboratory of J. King.

All phage used in these experiments carried the mutation c_{1-7}, which prevents lysogeny. Some also carried the mutations gene *13amH101,* which delays lysis, and gene *2amH202* or gene *3amN6,* either of which prevents DNA packaging (King *et al.,* 1973).

Media

LB medium was used to grow bacteria for plating experiments, complementation tests, and preparation of phage stocks. Superbroth (Fuller and King, 1981) was used to grow bacteria for the preparation of infected cell lysates and procapsid preparations. Dilution fluid (DF) used for serial dilutions of phage for titering was 0.1% tryptone, 0.7% NaCl, 2 m*M* $MgSO_4$.

Isolation of new mutants and crosses

Revertants of gene *8* amber mutants were selected by plating on the nonsuppressing host at 30°. These revertants were screened by stabbing onto plates incubated in parallel at 17, 30, and 39° for plaques that could not grow at high or low temperatures. Revertants and suppressors of temperature- and cold-sensitive mutations were selected by plating at 39 or 17° respectively, and screening the resultant plaques for inability to grow at the opposite temperature. Plaques displaying *ts* or *cs* phenotypes were grown up into stocks (Gordon and King, 1994) and retested. The mutations were assigned to gene *8* or gene *5* by liquid complementation tests. Those in gene *8* were mapped to one of four regions, AA, BB, CC, or DD, within the gene by recombination with mapping plasmids containing different P22 gene fragments as de-

scribed by Casjens *et al.* (1991). To cross the new mutants into amber backgrounds, exponentially growing DB7155 cells in LB at a concentration of 2×10^8/ml were infected with phage strains carrying desired alleles, each at a multiplicity of infection (m.o.i.) of five phage per cell. Progeny phage were screened for desired alleles.

DNA sequencing

DNA sequencing was performed as described in Gordon and King (1993). Briefly, a 1.5-kb region of phage DNA was amplified by symmetric PCR using primer 1, corresponding to nucleotides 3830–3854 and primer 2, complementary to nucleotides 5305–5276. The numbering is according to the notation of Eppler *et al.* (1991), in which the scaffolding coding region consists of nucleotides 3893–4801. The double-stranded DNA product was purified on an agarose gel, and the bands were cut out and cleaned using GeneClean (Bio101). This DNA was used as a template for synthesis of single-stranded DNA by asymmetric PCR using one or the other of the original primers. After purification with GeneClean, this DNA was used as the template for dideoxy DNA sequencing using the Sequenase kit (U. S. Biochemical). Primers used for sequencing were primer 3 (corresponding to nucleotides 4299–4315) and primer 4 (complementary to nucleotides 4426–4410) for sequencing region BB; primer 5 (corresponding to nucleotides 4675–4699) and primer 6 (complementary to nucleotides 4751–4727) for region CC. All primers were synthesized by the MIT Biopolymers Laboratory.

Preparation of cell lysates

Cells (7136) were grown to 4×10^8/ml in superbroth. Twenty milliliters of cells were each infected with a phage strain at an m.o.i. of 10. The infections were allowed to proceed for 3 hr at 30 or 39.5°, and for $5\frac{1}{2}$ hr at 17°. Cells were then concentrated by low-speed centrifugation and resuspended to 0.5 ml in buffer B (50 m*M* Tris; 25 m*M* NaCl; 2 m*M* EDTA, pH 7.6). The cells were lysed by three cycles of freezing and thawing. Lysates were cleared of DNA by addition of 20 m*M* $MgS0_4$ and 10 μg/ml DNase (Sigma).

Samples (200 μl) of lysates were loaded onto 5 ml 5–20% sucrose gradients, with 0.2-ml cushions of 50% $CsCl_2$/20% sucrose. The gradients were centrifuged for 35 min at 35,000 rpm in a Beckman SW50.1 rotor. Gradients were fractionated through a pinhole in the bottom of the tube into 18 fractions. Fractions were concentrated by TCA precipitation and then analyzed by SDS–PAGE.

Electron microscopy

Samples of cell lysates diluted to approximately 1 mg/ml were deposited onto carbon-coated copper grids, negatively stained by 2% uranyl acetate, and air dried. Grids were examined in a JEOL 1200 electron microscope at 80 kV. At least four micrographs were taken of each sample, and 150–250 particles were counted. The structures observed were classified into the following categories: Phage, electron-dense particles with an angular shape that usually have a tail at one corner; Procapsids, round particles with rough edges (with some internal density or empty; we did not try to count seperate classes of full versus empty procapsids); Empty heads, angular particles with smoother, thinner edges than procapsids and no internal density; Small, round particles that are approximately half the diameter of procapsids; Aberrant, spirals, figure 9s, and oversized or misshapen capsids.

Agarose gels

Samples of lysates or purified capsids were expanded *in vitro* by heating at 65° for 20 min (Galisteo and King, 1993). Expanded and unexpanded capsids were separated by electrophoresis on 1.8% agarose gels as described in Galisteo and King (1993).

Scaffolding extraction

Procapsids made by cells infected with mutant phage at the permissive temperature were purified as previously described (Prevelige *et al.,* 1988). Samples at 1.5 mg/ml were incubated overnight in varied concentrations of guanidine hydrochloride at 25°. The amount of scaffolding extracted at each concentration was quantified by separating free scaffolding from capsids by sucrose gradient sedimentation as described (Greene and King, 1994), except that only 6 fractions rather than 18 were collected from each gradient.

RESULTS

Isolation and sequencing of new mutants in gene *8*

Previous mutagenesis of the P22 genome over the course of two decades had resulted in the recovery of only two *ts* mutant alleles mapping to a single site in gene *8,* tsN102 and tsU172. Both of these represented the same alterations in the DNA sequence (M. Galisteo, unpublished results). By comparision, the same mutant searches had yielded over 20 *ts* alleles in the coat protein, gp5, and over 50 in the tailspike, gp9. If most of the scaffolding sequence was acting as a simple structural support rod, it might be less susceptible to *ts* lesions. Jarvik and Botstein (1975) found that selecting pseudorevertants or suppressors of existing conditional lethal mutations was an efficient way to find new *ts* and *cs* mutants. A set of amber mutants in gene *8* had been previously characterized (King *et al.,* 1978). The only known *cs* mutant in gene *8* had been selected as a psuedorevertant of a gene *8* amber (Jarvik and Botstein, 1975). We

TABLE 1

Selection of New Pseudorevertants and Second-Site Suppressor Mutations

Original mutant	Site of mutation	Revertant plaques screened	New mutants obtained
8amU237	unknown	175	None
8amU238	L226	200	None
8amU239	Q8	75	None
8amU240	Y214	200	8tsY214W
8amU241	Q154	150	None
8amH202	Q154	150	None
8amN26	Q149	100	8ts/csQ149W
5cs567	T10I	100	None
5cs577	N414S	100	None
5csU102	R101C	100	8tsL177I 5ts
8tsN102	S242F	100	None
8tsY214W	Y214W	100	None

adopted this strategy to select additional *ts* or *cs* mutants in gene *8.*

As shown in Table 1, many of the starting amber mutants did not yield any *ts* or *cs* pseudorevertants, perhaps because all substitutions at these sites are either permissive or lethal. Two amber sites did yield *ts* pseudorevertants, one at the site of the original *cs* pseudorevertant; this mutation had both *cs* and *ts* phenotypes. We also attempted to select gene *8* mutants as suppressors of mutations in gene *5,* on the assumption that the coat and scaffolding proteins must interact. Although there are many *ts* coat alleles, all have the phenotype of interfering with coat polypeptide chain folding, prior to interaction with scaffolding (Gordon and King, 1993). They did not seem likely candidates to be suppressed by scaffolding mutants. Instead we sought suppressors of three coat *cs* mutations, which appeared to be better candidates for affecting coat/scaffolding interactions.

The gene *5* mutation *csN414S* causes production of procapsids containing scaffolding that do not package DNA and are blocked in expansion *in vitro* (Gordon, Lee, Reiner, and King, unpublished results). The two N-terminal mutations, *csT10I* and *csR101C,* result in procapsids that have lost most of their scaffolding. (Gordon, Lee, Reiner, and King; Greene and King, unpublished results). Only the coat *cs* mutant *R101C* yielded any second site suppressors in gene *8* with a *ts* phenotype. The new strain bearing the suppressor was crossed to an amber in gene 5 and backcrossed to wild-type so as to eliminate the parent *cs* mutation.

The new mutants were assigned to gene *8* by liquid complementation tests with both gene *5* and gene *8* amber mutants, and then mapped to specific regions of gene *8* by recombination with both gene *8* ambers at known locations and with mapping plasmids carrying pieces of the P22 genome (Casjens *et al.,* 1991). Regions of gene *8* containing the new mutations as well as the original amber and gene *5 cs* mutants were sequenced to determine the amino acid changes. The results are shown in Table 2.

There are five mutants at four different sites (Fig. 1). The locations of the changes are consistent with the locations of the parent amber mutations and with the genetic mapping. The strains with mutations *Q149Y* and *Q149W* also carry the mutation *A199V.* Since this change is also present in the parent amber strain, which is neither *ts* nor *cs* on the Gln-inserting supressor host, this appears to be a silent mutation. In most cases the change results in the substitution of a bulky aromatic residue for a smaller sidechain which may result in destabilization of the protein fold. The one exception, *L177I,* is a more conservative substitution, but the β-branched residues such as isoleucine are known to destabilize α helices (Cornish *et al.,* 1994).

Phenotypes of mutants *in vivo*

In order to determine the effects of these mutations on the phage assembly pathway, cells were infected with mutant strains at both permissive and nonpermissive temperatures. The resulting lysates were clarified and concentrated. Lysates were examined by electron microscopy, to observe any large structures accumulated, and by sucrose gradient sedimentation and SDS–PAGE, to determine the protein composition of these structures. This approach assumes that some structures will in fact be made, but this assumption seemed reasonable given that even in the absence of scaffolding protein, as in a gene *8* amber infection, the coat subunits can form some aberrant large assemblies (King *et al.,* 1973; Earnshaw and King, 1978).

At the permissive temperature of 30°, all the mutants were similar to wild-type, producing both procapsids and infectious phage. At the nonpermissive temperature, all the mutants were capable of assembling with coat protein into large structures, visible in the micrographs and as rapidly sedimenting peaks on the sucrose gradients. The amount of coat protein assembled into structures appeared roughly comparable to wild-type, indicating that the mutants had not lost the ability to associate with the coat protein. However, the capsids produced by each mutant exhibited specific defects.

At 40°, the wild-type strain produced procapsids and infectious phage (Fig. 3A). The phage are the more angular, electron-dense particles with tails. The procapsids are rounder with some stain penetrating the shell. The sucrose gradient revealed a peak at the bottom, of the heavy, DNA-containing phage, and another around fractions 4–6, containing the procapsids (Fig. 2a). The procapsid peak includes five proteins: coat, scaffolding, portal, and the two pilot proteins gp16 and gp20. The third pilot protein gp7 is too small to be detected on these gels.

TABLE 2

Sequence Changes in Temperature- and Cold-Sensitive Mutants in the P22 Scaffolding Gene

Mutant	Nucleotides sequenced	Nucleotide substitution	Amino acid substitution	Local amino acid sequence
8ts N102	505–800	C765T	Ser242→Phe	ELTRLSERLTL
8ts pseuodorevertant of 8am U240	465–680	A641G T642G	Tyr214→Trp	SAALMYHLGAN
8ts suppressor of 5csU102	505–800	C445T	Leu177→Ile	DAAEKLNIPDY
8ts/cs pseudorevertant of 8amN26	355–665	C445T A446G C596T	Gln149→Trp Ala199→Val	NAVAEQGRKTQ
8cs pseudorevertant of 8amN26	335–800	C445T G447T C596T	Gln149→Tyr Ala199→Val	NAVAEQGRKTQ
8amU240	505–800	T642G	Tyr214→amber	
8amN26	225–665	C445T C596T	Gln149→amber Ala199→Val	
5csU102	215–485	C301T	Arg101→Cys	DETAYRRRIQS

None of the *ts* mutants produced any infectious phage, with burst sizes reduced over 100-fold (Table 3). The mutant *S242F* had been previously characterized by Bazinet and King (1988). Consistent with their results, this mutant produced procapsids that contained only coat and scaffolding. These structures failed to include the portal or the products of genes 16 and 20. These procapsids also included less than the usual amount of scaffolding protein and were therefore somewhat lighter, sedimenting more slowly on the sucrose gradient than wild-type procapsids (Fig. 2b). The procapsids produced by this mutant appeared morphologically normal (Fig. 3B).

The mutant *Y214W* had a similar phenotype, also yielding morphologically normal procapsids that lacked the portal. However, these capsids did contain the usual amounts of gp16 and 20 and appeared to have the full complement of scaffolding protein, thus sedimenting slightly faster than the *S242F* procapsids (Fig. 2c).

The mutant *tsL177I* accumulated procapsids that lacked only gp16 (Fig. 2d). This mutant lysate also contained many particles which resembled mature, tailed phage (Fig. 3D). These particles were not infectious, since the number of plaque-forming units in this lysate was reduced a 1000-fold compared to wild-type. Presumably, the phage also lacked gp16 and were thus incapable of infection. The amount of phage produced, however, was much less than in the wild-type infection. While the cells infected with the wild-type strain were filled primarily with mature phage at the end of the infection (Fig. 4) those infected with the mutant *L177I* still contained mostly procapsids, suggesting that this mutant caused an inhibition of maturation.

Mutant *Q149W* is both temperature and cold-sensitive. At high temperature (40°), this mutant produced some normal appearing procapsids, but also many that were incorrectly formed. As seen in Fig. 3E, these particles appeared distorted in shape rather than round. The morphological defect produced particles both longer and wider than usual. Some of these structures were not closed, but resembled figure 9s. This phenotype was different from that produced in the absence of scaffolding protein, as in a gene *8* amber infection. The structures produced in the *8am* infection were far more severely aberrant spirals containing multiple whorls (Fig. 3F). Also, the *8am* infection produced round particles smaller in size than procapsids, which were not seen in the *Q149W* lysate (Fig. 4). In addition, while the structures produced in the *8am* infection contained only coat protein, those produced by *Q149W* appeared to contain portal protein, which is visible across the gradient in proportion to the coat protein present (Figs. 2e and 2f). The *Q149W* lysate also contained some noninfectious phage particles. As with *L177I,* the amount of phage produced was lower than for wild-type (Fig. 4).

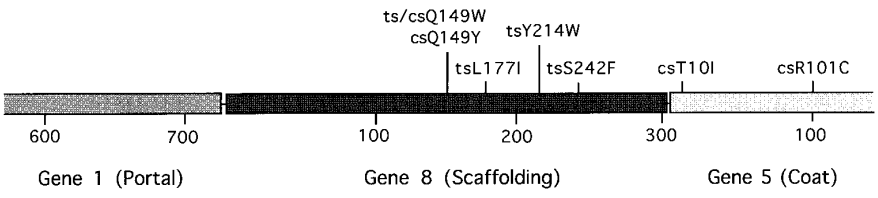


FIG. 1. Location of conditional lethal mutations within the bacteriophage P22 scaffolding gene.

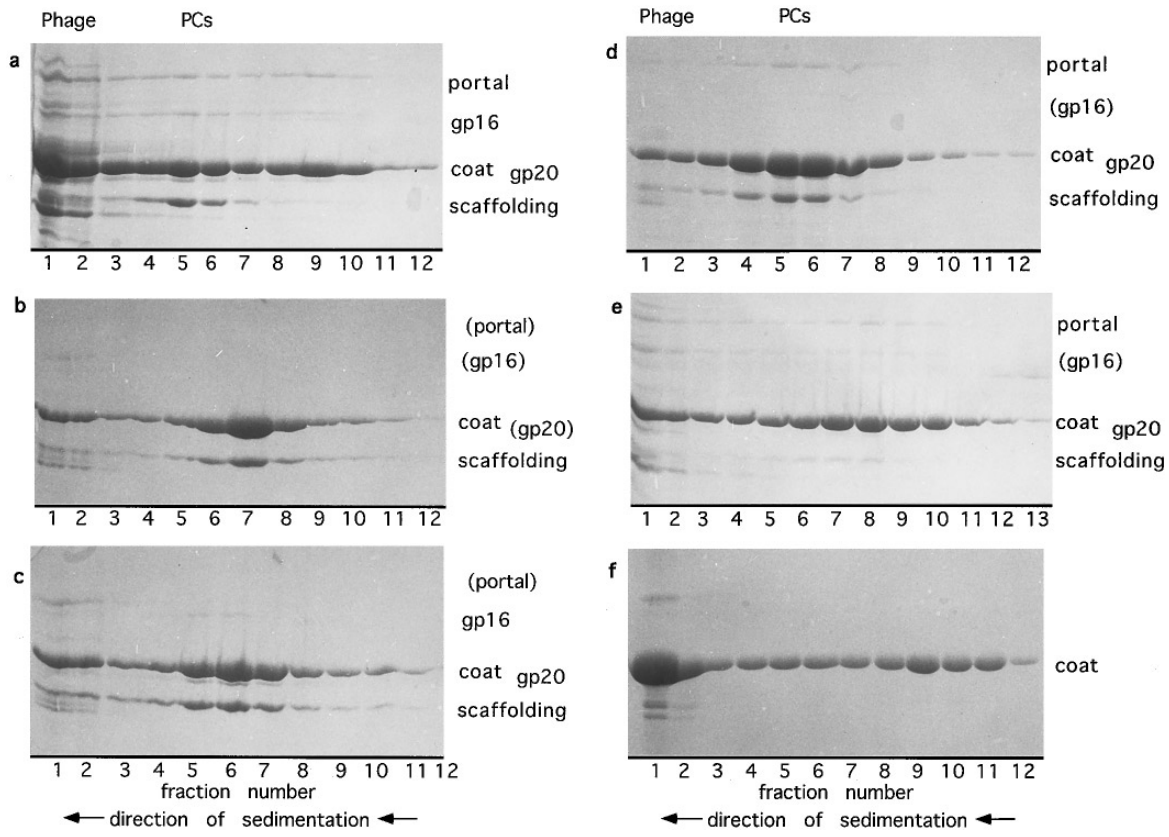


FIG. 2. Sucrose gradients of lysates from cells infected with temperature-sensitive scaffolding mutants at 40°. Samples (200 μl) of lysates prepared as described under Materials and Methods were centrifuged through 5–20% sucrose gradients. 18 fractions were collected from each gradient and their protein composition was analyzed by SDS–PAGE. The positions to which wild-type phage and procapsids sediment on the gradient are indicated at the top of the figure. Procapsid proteins not present within a given gradient are marked with brackets. Structures made in the scaffolding amber infection, shown as a control, contain only coat protein. (a) Wild-type, (b) 8ts S242F, (c) 8ts Y214W, (d) 8ts L177I, (e) 8ts/cs Q149W, (f) 8 amber.

Both substitutions at Q149 exhibited a cold-sensitive phenotype, although this was not as strict a defect as the *ts* phenotype; the burst size was reduced only by a factor of 10 at 17° (Table 3). Both substitutions had a similar phenotype: procapsids of normal dimensions were produced, but many lacked scaffolding protein, as seen in fractions 9–10 on the sucrose gradients (Fig. 5) and by their empty appearance in the electron micrographs (Fig. 6). The empty capsids did not appear to have undergone expansion, but displayed the round shape and rough edges typical of unexpanded procapsids.

TABLE 3

Burst Sizes of Scaffolidng Mutant Strains

	Phage per Infected Cell		
Infecting phage	30°	40°	17°
Wild-type scaffolding	265	525	189
Amber	<2	<2	
8tsS242F	248	<2	
8tsY214W	333	<2	
8tsL177I	470	3	
8ts/csQ149W	248	2	38
8csQ149Y	229		16

Phenotypes of mutants with DNA packaging blocked

The inability of the *S242F* and *Y214W* procapsids to progress to mature phage was readily explained by the fact that these capsids did not include portals, thus precluding the possibility of DNA packaging. It was more difficult to explain why the *L177I* procapsids were inhibited in maturation, since the presence of gp16 is not required for either assembly or DNA packaging (Botstein *et al.,* 1973; Bryant and King, 1984). It was also not clear whether the procapsids made by the cold-sensitive mutants had lost their scaffolding subsequent to abortive DNA packaging attempts or whether the premature exit of scaffolding in some way hindered DNA packaging. To address these issues, all of the mutations were crossed with an amber mutation in gene *2,* whose product is required for DNA packaging; thus, these strains will all accumulate procapsids. As expected, all strains produced procapsids of normal composition at 30°.

At 40°, the *ts* mutants all produced procapsids, whose protein compositions are shown in Fig. 7. With DNA pack-

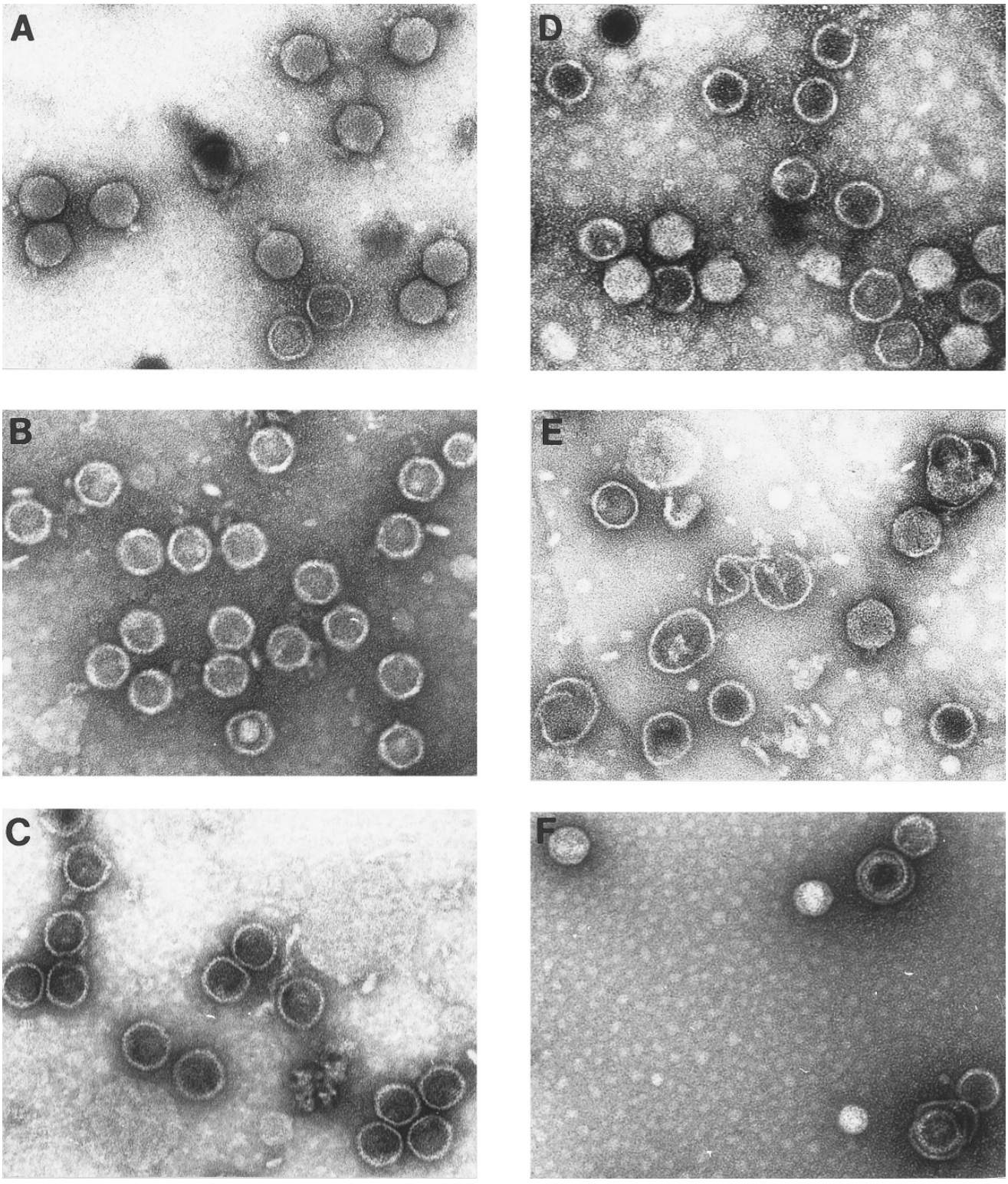


FIG. 3. Electron microscopy of structures produced by gene 8 ts mutants at 40°. The lysates subjected to sucrose gradient centrifugation shown in Fig. 2 were observed by negative stain at a magnification of 60,000×. (A) Wild-type, (B) 8tsS242F, (C) 8ts Y214W, (D) 8ts L177I, (E) 8ts/cs Q149W, (F) 8 amber.

aging blocked, the phenotypes of *S242F* and *Y214W* at 40° were the same as previously observed, as expected, since these capsids cannot package DNA in the wild-type background. The double mutant *L177I/2am* produced only procapsids, which lacked gp16. The lysate of the double mutant *Q149W/2am* at 40° contained a

higher proportion of morphologically normal procapsids than observed in the absence of the DNA packaging block. These particles were presumably the precursors to the noninfectious phage particles observed in the wild-type background. It was apparent from SDS–PAGE that these particles did not contain gp16, as with *L177I,* revealing why they cannot mature into infectious phage.

Even with DNA packaging blocked, the cold-sensitive mutants still accumulated many empty capsids at low temperature, demonstrating that the scaffolding had not been released by initiation of DNA packaging. The sucrose gradients shown in Fig. 8 indicated a mixed population of particles, some of which contained more scaffolding, suggesting that all procapsids may have originally contained scaffolding, but that it had prematurely "leaked" from many. These capsids also contained decreased amounts of gp16 and gp20, but as these proteins are easily released by conditions which extract scaffolding it is more likely that they have leaked out of the assembled capsids than that the capsids were assembled without them. It is not clear why the loss of scaffolding protein should prevent DNA packaging, as the scaffolding must normally be released in any event. This phenotype is the same as that observed for the two *cs*

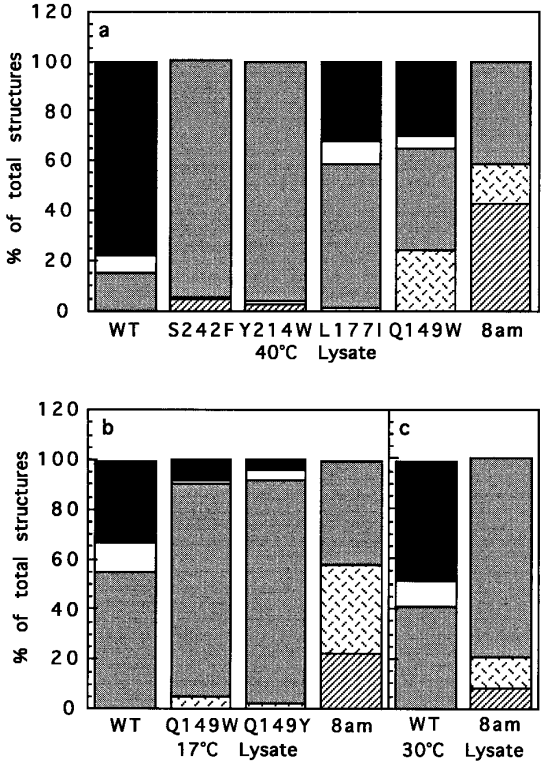


FIG. 4. Structures produced by scaffolding mutant strains at nonpermissive temperature. The structures observed in electron micrographs of infected cell lysates were counted and classified according to the criteria described under Materials and Methods. (a) Lysates from cells infected at 40°, (b) 17°, (c) 30° controls. Black, phage; white, empty heads; gray, procapsids (or empty procapsids); crosshatching, aberrant structures; stripes, small capsids.

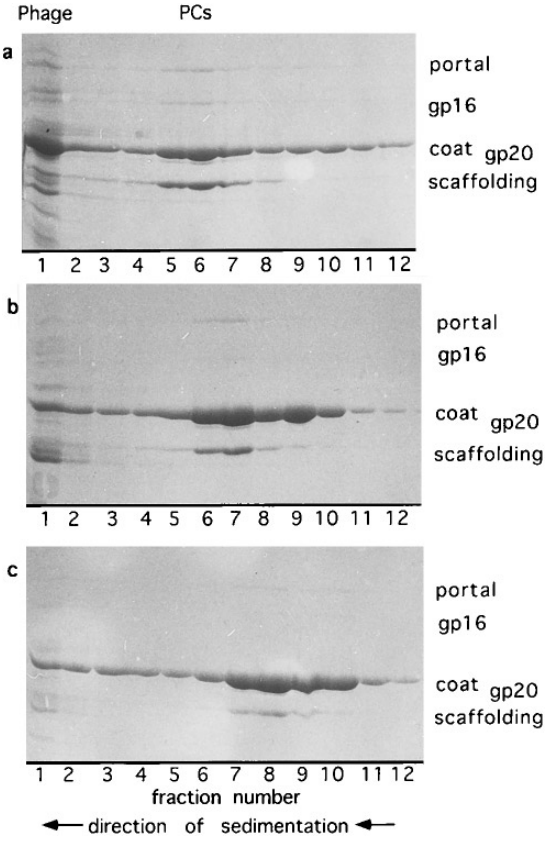


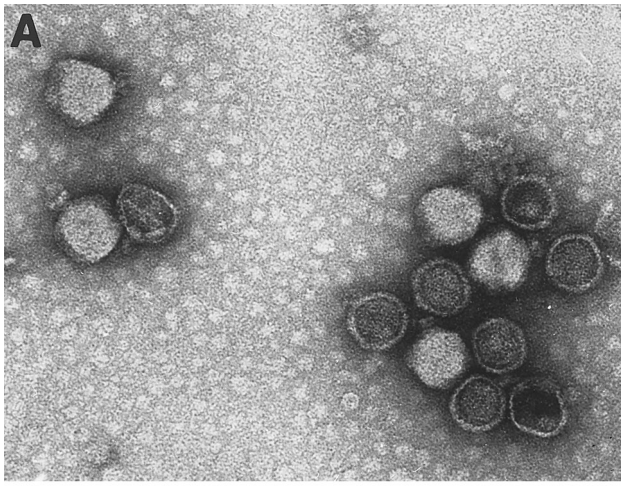
FIG. 5. Sucrose gradients of lysates from cells infected with cold-sensitive scaffolding mutants at 17°. Samples (200 μl) of lysates prepared as described under Materials and Methods were centrifuged through 5–20% sucrose gradients. 18 fractions were collected from each gradient and their protein composition was analyzed by SDS–PAGE. The positions to which wild-type phage and procapsids sediment on the gradient are indicated at the top of the figure. Empty shells trail procapsids. (a) Wild-type, (b) 8ts/cs Q149W, (c) 8cs Q149Y.

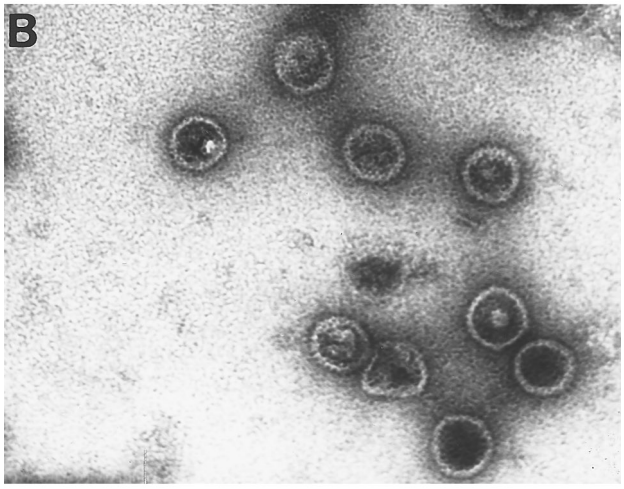
mutants in the N-terminal region of the coat protein (Gordon, Lee, Reiner, and King; Greene and King, unpublished results).

Scaffolding release

Since we were unable to explain the inabilty of some of the mutants to package DNA, we decided to test *in vitro* their ability to undergo the processes normally coupled with DNA packaging, such as the expansion of the procapsid to the mature capsid form and scaffolding release. Guanidine hydrochloride at only 0.5 *M* is sufficient to extract all the scaffolding from wild-type procapsids (Prevelige *et al.,* 1988; Greene and King, 1994). Procapsids were purified from cells infected with the five double mutant strains blocked in DNA packaging. The infections were carried out at the permissive temperature, so that the procapsids synthesized by the five mutant strains all had equivalent protein compositions. Scaffolding was extracted from these isolated procapsids by incubation in a range of

GuHCl concentrations at room temperature. It was not possible to perform this experiment with *Q149Y* procapsids, as much of the scaffolding was already lost by the end of the purification process, which involved many steps at 4°.





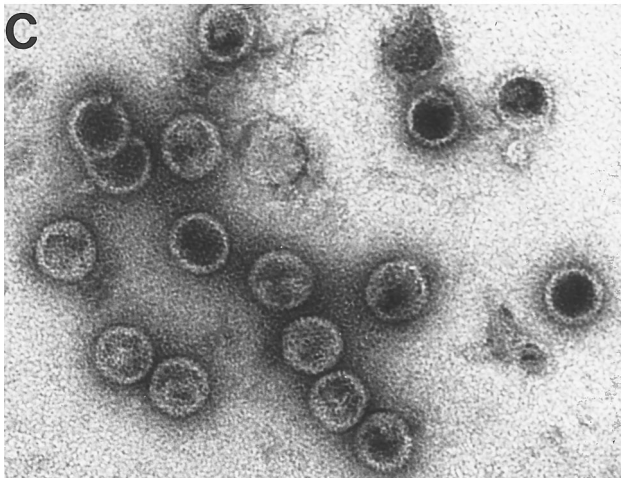


FIG. 6. Electron microscopy of structures produced by gene *8* cs mutants at 17°. The lysates subjected to sucrose gradient centrifugation shown in Fig. 5 were observed by negative stain at a magnification of 60,000×. (a) Wild-type, (b) 8cs/ts Q149W, (c) 8cs Q149Y.

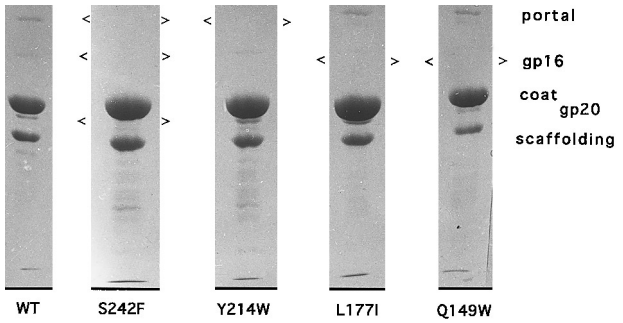


FIG. 7. Protein composition of procapsids produced by *8ts* mutants at 40° with DNA packaging blocked. Lysates of cells infected with *8ts/2am* double mutant strains were prepared and centrifuged through 5–20% sucrose gradients. The gradients were fractionated and the location of the capsid peaks was determined by SDS–PAGE. Only the peak fractions are shown here. The positions of proteins absent from mutant procapsids are indicated by brackets.

As shown in Fig. 9, the extraction of scaffolding from procapsids made by *S242F* and *Y214W* at permissive temperature had a profile similar to that observed for wild-type. Extraction of *L177I* procapsids was markedly different; even 1.0 *M* GuHCl was insufficient to release more than about half of the scaffolding. The difference for *Q149W* was less dramatic, but this mutant scaffolding was still clearly more difficult to release than wild-type, an unexpected result given its apparent leakiness at low temperature.

Procapsid to capsid expansion *in vitro*

The procapsids of all the dsDNA phages expand radially in forming the mature shell. Procapsid expansion *in vivo* is coupled to packaging of the DNA. The expansion can be triggered *in vitro* in the absence of DNA by a variety of treatments, including heat. The expansion is conveniently monitored by electrophoresis through agarose gels. This *in vitro* expansion of procapsids to the mature form is an exothermic reaction when followed by calorimetry (Galisteo and King, 1993). After heating at 65° for 20 min, about half of the wild-type procapsids were found to undergo expansion, as observed by migration through an agarose gel (Fig. 10a). The degree of expansion was similar regardless of the temperature at which the wild-type procapsids had been produced.

The mutant procapsids fell into three classes with respect to the expansion reaction: *L177I,* which failed to expand; *Q149Y/W,* which expanded more readily than wild-type, and *S242F* and *Y214W,* which displayed conditional expansion.

The procapsids of *L177I* produced at both temperatures did not expand, indicating that some property of this mutant scaffolding trapped the coat lattice in the unexpanded state.

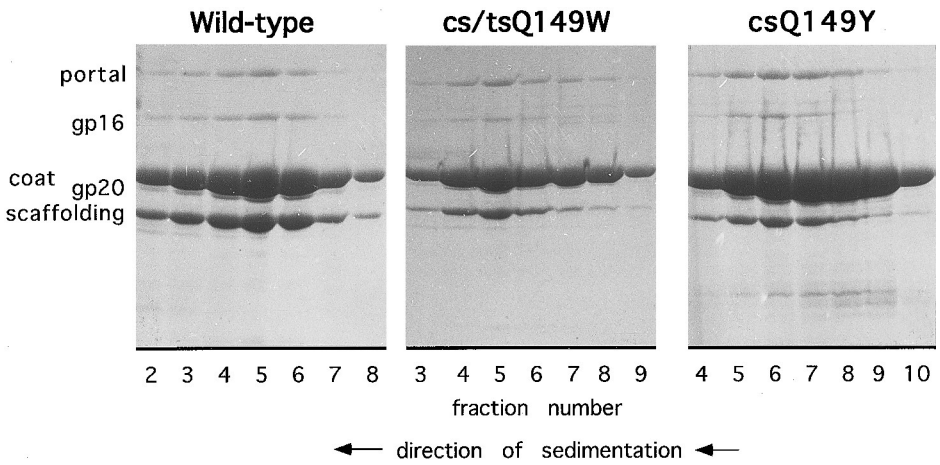


FIG. 8. Protein composition of procapsids produced by *8cs* mutants at 17° with DNA packaging blocked. Lysates of cells infected with *8cs/2am* double mutant strains were prepared and centrifuged through 5–20% sucrose gradients. 18 fractions were collected from each gradient and their protein composition was determined by SDS–PAGE. Fractions including the procapsid peaks from each gradient are shown.

The procapsids made by mutants *Q149W* and *Q149Y* not only expanded, but expanded more readily than wild-type procapsids, as no unexpanded capsids were left after 20 min of heating (Fig. 10b). This property did not depend on the temperature at which the mutant procapsids were made. The unheated particles migrated to the same position as procapsids, demonstrating that the empty particles produced at low temperature were not already expanded. Procapsids made with the two N-terminal coat *cs* mutants, including the one suppressed by *L177I,* also expanded more completely than wild-type (not shown).

The *Q149Y/W* procapsids which expanded more readily than wild-type also leaked scaffolding at low temperature. In contrast, the capsids produced by *L177I* contained more tightly bound scaffolding and did not expand. We suspected that release of scaffolding might be a prerequisite for procapsid expansion, so that capsids from which scaffolding was more easily released would also expand more readily.

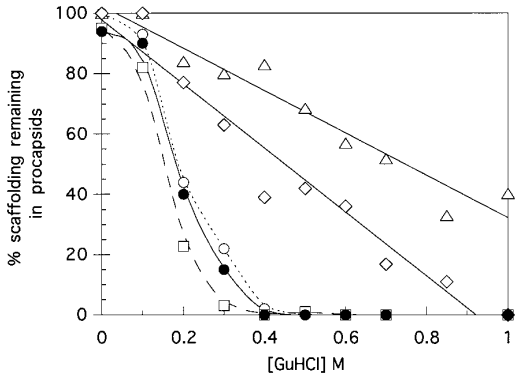


FIG. 9. GuHCl-induced extraction of mutant scaffolding proteins from procapsids. Procapsids purified from cells infected with *2am* or *8ts/2am* double mutant strains at the permissive temperature were incubated at 1.5 mg/ml in varied concentrations of GuHCl. Extracted scaffolding was separated from procapsids by sucrose gradient sedimentation. The protein composition of the gradient fractions was analyzed by SDS–PAGE. The amount of scaffolding remaining within the capsids and that sedimenting as monomers were quantified by densitometry of the Coomassie stained gels. Filled circles, wild-type; open circles, S242F; squares, Y214W; triangles, L177I; diamonds, Q149W.

To test this idea we extracted the scaffolding from mutant procapsids made at the permissive temperature, to yield shells containing only coat protein. If the aberrant expansion properties were due to differences in the extraction of the scaffolding proteins by heat, then the empty shells should all demonstrate the same expansion characteristics. This was not the case; the shells derived from *L177I* procapsids did not expand, while those from *Q149W* and *Q149Y* still expanded more completely than wild-type shells (Fig. 10c). It was not possible to fully extract all the scaffolding from the *L177I* procapsids even after multiple extractions with GuHCl, so the failure of these shells to expand might be due to residual tightly bound scaffolding. Nonetheless the *Q149Y/W* results suggest that the mutant scaffoldings might alter the coat lattice or the packing of the portal ring into the coat lattice.

The procapsids produced by the mutant *S242F* at 30° underwent expansion to the same degree as wild-type. However, procapsids carrying *S242F* formed at 40° failed to expand in the gel assay. The same permissive and restrictive phenotypes were observed for the mutant *Y214W.* Since the procapsids produced by both these mutants lack portals at the high temperature, it was possible that the portal was needed for the expansion reaction.

To test this possibility, procapsids were isolated from cells infected with phage carrying an amber mutation in gene *1,* the portal protein gene. The N-terminal portal fragment produced in these cells is not incorporated into the procapsids assembled in these cells. These procapsids composed of wild-type scaffolding protein but lack-

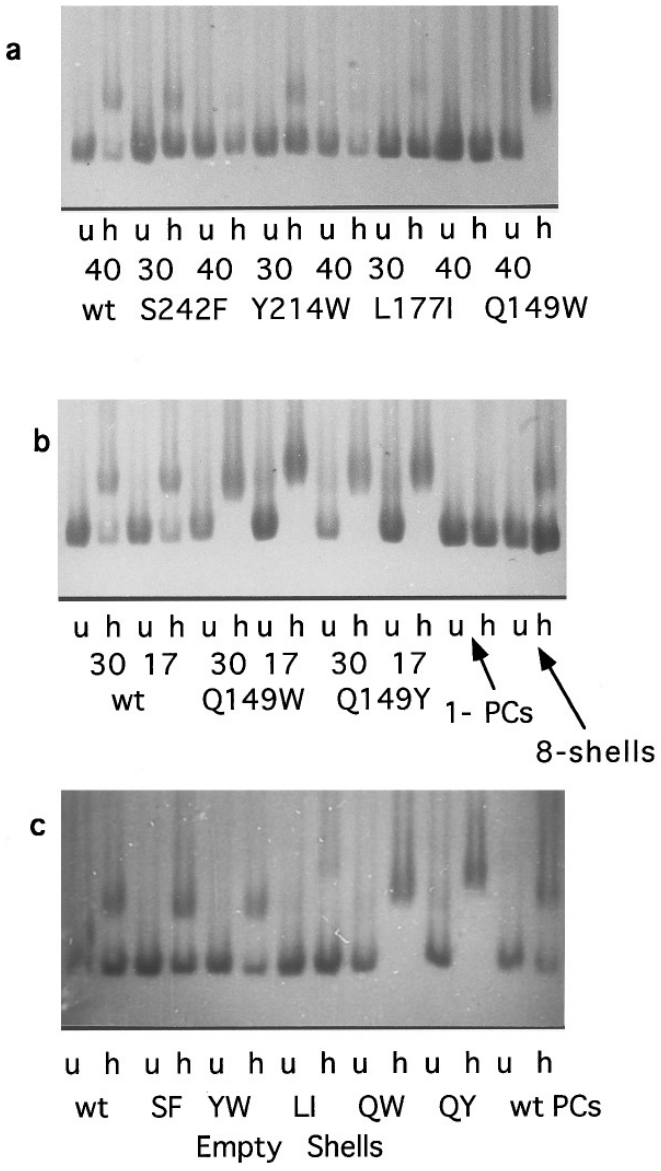


FIG. 10. *In vitro* expansion of wild-type and mutant procapsids and shells. Procapsid and shell samples at 1 mg/ml were either untreated (u) or heated (h) for 20 min at 65°. Samples were mixed with Serwer sample buffer and the expanded and unexpanded capsids were separated by electrophoresis though 1.8% agarose gels. (a and b) Lysates of cells infected with *8ts/2am* mutants at the designated temperature; (c) purified shells with scaffolding extracted.

ing portal rings failed to expand *in vitro,* suggesting that the portal was required for the initiation or propagation of expansion.

We also examined the behavior of the normally sized capsids lacking the portal and other minor proteins ("*8*$^{-}$ shells") that are assembled in cells infected with phage bearing an amber mutation in the scaffolding gene (Earnshaw and King, 1978). Although not containing portals, these shells expanded to the same extent as wild-type procapsids. This result suggested that the expansion reaction does not require a portal structure.

This paradox may be resolved by noting that the *8*$^{-}$ shells differ from the portalless procapsids in lacking scaffolding. The inability of *L177I* procapsids to expand and the failure to observe expanded capsids containing scaffolding in any system imply that scaffolding release may be a prerequisite for capsid expansion. The presence of the portal might be required to initiate not expansion itself, but scaffolding release. This would explain previous observations that higher GuHCl concentrations are required to extract wild-type scaffolding from portalless procapsids and that this extraction process is less cooperative than that observed with procapsids containing portals (B. Greene, unpublished results). In this case, while lack of a portal would block expansion by impairing scaffolding release, the absence of both portal and scaffolding would not affect expansion.

DISCUSSION

Isolation of new missense mutants in the scaffolding gene

New mutants were successfully isolated by screening revertants of existing amber and missense mutations as described by Jarvik and Botstein (1975). Another strategy would have been extensive mutagenesis of a local region, as has recently been done to isolate mutations affecting four different functions of yeast calmodulin (Ohya and Botstein, 1994). However, these protocols are typically guided by the availability of the three-dimensional structure and prior knowledge of the functional domains, information that was not available for the P22 or any other scaffolding protein. The failure to obtain mutants in the scaffolding gene by random mutagenesis suggested that many sites might be either highly tolerant or intolerant to substitution. Any targeted mutagenesis of the 303-amino-acid scaffolding protein would thus probably have a very low likelihood of generating useful substitutions. The strategies used here allowed targeting of the scaffolding gene, unlike a general mutant hunt, while the screens used to identify mutant phage guaranteed that any mutants obtained would have a phenotype.

Temperature-sensitive mutations in both the tailspike and coat protein shift the chainfolding pathway from productive folding to aggregation (Haase-Pettingell and King, 1988; Gordon and King, 1993). At high temperature, the *ts* coat mutants do not form any regular structures, but aggregate into inclusion bodies (Gordon and King, 1993). This does not appear to be the case with the scaffolding *ts* mutants, suggesting that their folding is less thermolabile. Another possible phenotype would have been scaffolding proteins completely unable to associate with coat protein. Yet all these mutants have clearly different phenotypes from the scaffolding amber strain, and all are found within coat particles. It appears that the mutants do selectively disrupt only particular functions of the protein.

Incorporation of minor proteins

A critical role for scaffolding protein in the incorporation of the portal and the pilot proteins had previously been inferred from the absence of minor proteins from the aberrant coat structures formed in scaffolding amber infections (Earnshaw and King, 1978), as well as from the phenotype of *S242F* (Bazinet and King, 1988). This evidence suggested that the scaffolding was a key component of an initiation complex that recruited the portal and pilot proteins.

The role of gp16 in procapsid assembly has been studied by Prevelige and Thomas (1991), who found that its presence accelerated the rate of initiation of *in vitro* procapsid assembly. Evidence from cosedimentation studies suggested that gp16 could interact with the coat protein, but not the scaffolding protein. Perhaps the exclusion of gp16 from *L177I* and *Q149W* procapsids is a secondary effect, rather than an indication of a defect in direct scaffolding/gp16 bonding.

It seems reasonable to suggest that the mutations *S242F* and *Y214W,* less than 30 amino acids apart in the sequence, affect a region involved in portal insertion, either by direct interaction with portal protein or by participation in an initiation complex that recruits the portal. Genetic evidence from lambda (Murialdo and Becker, 1978b; Murialdo, 1979) and T4 (Laemmli *et al.,* 1970) indicates that scaffolding is required for the first initiation steps, in which the portal vertex is assembled.

Direct interaction between the portal and scaffolding proteins of phi29 was observed by gel shift assays (Guo *et al.,* 1991). An amber mutation at position 172 of the 269 amino acid gp22 in T4 has a *ts* phenotype on a tyr-inserting host; a second site suppressor of this mutation maps to gene 20, encoding the portal (Mesyanzhinov *et al.,* 1990). The authors proposed that this mutant identified a region of the protein, modeled as an α helix, that interacted with the portal. This T4 mutant is in the C-terminal third of the protein, as are the P22 mutants defective in portal insertion. It seems likely that one essential role of phage scaffolding proteins is to incorporate the portal, and these mutations may identify the specific region in the P22 protein responsible for this activity, a region which may be conserved in other scaffolding proteins.

Morphogenesis

The aberrant shells of *Q149W* at high temperature are reminiscent of defects produced by temperature-sensitive mutants of the major T4 scaffolding protein, gp22 (Keller *et al.,* 1988). These mutants generated procapsids of aberrant shapes that could be described as perturbations of the basic icosahedral symmetry (Kellenberger, 1990); some were isometric rather than prolate, or prolate but of insufficient length, while others were bi- or triprolate. P22 procapsids are isometric and so do not need as complicated a sizing mechanism as T4. The P22 aberrant capsids do not appear to have any recognizable pattern of defects; they may not even be closed shells, but related to spirals. Unlike spirals, however, they appear to have included portals, showing that portal insertion and elongation are separable functions. In contrast to phi29, in which the portal seems to determine the correct length of the prolate capsid (Guo *et al.,* 1991), these mutant procapsids can insert the portal correctly, but still assemble incorrectly, demonstrating that scaffolding plays the essential controlling role. However, these capsids are less defective than structures produced in scaffolding amber infections, perhaps indicating some role for initiation in controlling assembly.

We think it is more likely that the presence of the long scaffolding molecules within the growing capsid provide a steric constraint that prevents the coat protein from forming the more tightly wound spirals found in the scaffolding amber lysates. In addition, the elongated scaffolding molecules might be making longer range contacts that drag the edges of the growing shell into forming a closed, although oddly shaped, capsid. Computer models of P22 procapsid assembly demonstrate that spirals can result from the insertion of a single hexamer in place of a pentamer (Berger *et al.,* 1994). By allowing the coat protein subunits to look further away for nearby subunits (as the addition of scaffolding molecules might allow), the structures could be made to close rather than spiral (B. Berger, personal communication).

Scaffolding release

The mutations at sites 177 and 149 affect the process of scaffolding extraction *in vitro.* If the scaffolding is also more difficult to release *in vivo,* that could account for the inefficiency of the procapsids produced by these mutants in packaging DNA.

What is the mechanism of this defect? The sites of these mutations may be part of a domain involved in binding to the coat protein. By altering residues that contact the coat protein, the mutations might make the binding surface "stickier" at high temperature. However, scaffolding constructs beginning at residue 180 are able to assemble coat subunits into procapsids (S. Casjens, personal communication), which makes it difficult to see how the region from 149 to 177 could be an essential coat-binding domain.

This region might instead mediate a scaffolding/scaffolding interaction. Since the scaffolding subunits probably must be monomeric in order to fit through the exit channels, substitutions that cause the mutant scaffolding proteins to associate more tightly would prevent them from exiting the procapsid.

Another possibility is that the release of scaffolding is not just a cessation of binding, but an active process in its own right, involving a new functional region. In this

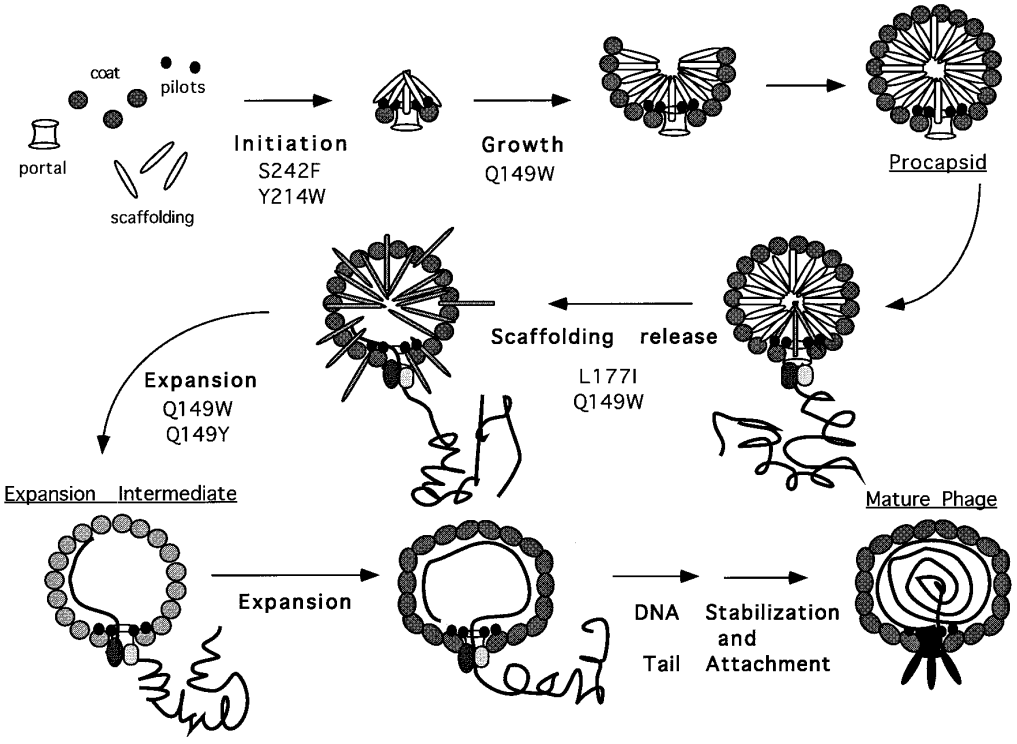


FIG. 11. An expanded pathway for the assembly of phage P22.

model, the docking of the DNA packaging complex to the portal would transmit a signal to the nearby scaffolding molecules. The receipt of this signal would induce a conformational change that would propagate throughout the capsid and cause the scaffolding subunits to cease binding. Some active switch would seem to be required, since the scaffolding in procapsids is normally stable and does not spontaneously diffuse out. The L177I mutant may be defective in this "release switch" so that the switch is not well triggered, or the signal not propagated. The scaffolding may diffuse out of the cs mutants because their switch is too easily triggered.

Capsid expansion

It is still unclear why the cold-sensitive mutants do not package DNA, since the release of scaffolding protein is a normal part of the wild-type maturation pathway. Models in which scaffolding release is energetically coupled to DNA packaging have been proposed, but are not overly convincing, as related phages, such as lambda and T4, can package DNA into capsids empty of scaffolding (Earnshaw and Casjens, 1980; Black, 1989). The *cs* mutant capsids are clearly able to continue on the maturation pathway by undergoing expansion; in fact, they expand more readily than wild-type. That two coat protein mutants share this phenotype supports the idea that it is due to differences in the structures of the capsids. The differences must be subtle, since the capsids tested were made at the permissive temperature and presumably would be able to mature into phage *in vivo.* Perhaps these capsids are a small step further along the expansion pathway than wild-type and so have prematurely passed the stage at which they are competent for DNA packaging.

An expanded pathway for P22 assembly

A more detailed model of the P22 assembly pathway is shown in Fig. 11, with the likely points of the mutant defects indicated. In this picture, the first assembly step is the formation of an intitiation complex containing scaffolding and the minor proteins, and perhaps the first few coat subunits. Extension of the capsid depends upon different scaffolding regions that control the recruitment of new coat subunits and the manipulation of their curvature to form a closed shell. This process probably involves two different scaffolding sites, one to bind the coat protein and another to bind the scaffolding subunits to each other, thus pulling the coat subunits into a closed capsid. Finally, scaffolding release is a distinct process, in which the docking of the DNA packaging complex at the portal triggers a "release switch" in nearby scaffolding subunits. This conformational change is propagated thoughout the scaffolding core so that all the scaffolding molecules cease to bind the coat and exit through the channels. Freed of scaffolding, the coat lattice is able to expand and close the channels. The capsid may then proceed through one or more intermediate states before reaching the final expanded conformation.

Viruses are often perceived from the static perspective of a high-resolution mature stucture. In this model, we hope to stress the dynamic nature of the assembly process. It is generally recognized that structural proteins must undergo conformational changes during assembly. The results of this paper indicate that associated assembly proteins are not passive support beams or templates, but also undergo active transformations.

ACKNOWLEDGMENTS

The authors thank Dr. Sherwood Casjens for the gift of cell strains carrying the P22 mapping plasmids, Morgan Kelly for isolation of the new mutant Y214W, and Raka Mustaphi for carrying out the *in vitro* extraction of mutant scaffoldings from procapsids. These investigations were supported by NIH Grant GM 17,980 to J.K., and fellowships from the W. M. Keck Foundation and the NIH Biotechnology Training Grant to B.G.

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